Journal of
Experimental
Botany

www.jxb.oxfordjournals.org

RESEARCH PAPER

Developmental onset of reproductive barriers and associated proteome changes in stigma/styles of *Solanum pennellii*

Subbaiah C. Chalivendra[1,4,*], Gloria Lopez-Casado[2,6,*], Aruna Kumar[3,5], Alina R. Kassenbrock[1], Suzanne Royer[1], Alejandro Tovar-Mèndez[3], Paul A. Covey[1], Laura A. Dempsey[1], April M. Randle[1], Stephen M. Stack[1], Jocelyn K.C. Rose[2], Bruce McClure[3] and Patricia A. Bedinger[1†]

[1] Department of Biology, Colorado State University, Fort Collins, CO 80523-1878, USA
[2] Department of Plant Biology, 412 Mann Library Building, Cornell University, Ithaca, NY 14853, USA
[3] Department of Biochemistry, University of Missouri, Columbia, MO 65211, USA
[4] Present address: Valent BioSciences Corporation, Long Grove, IL 60047, USA
[5] Present address: Amity Institute of Biotechnology, J3 block, Sector-125, Noida, Uttar Pradesh 201303, India
[6] Present address: Instituto de Hortofruticultura Subtropical y Mediterránea (IHSM) 'La Mayora', Centro Mixto CSIC-Universidad de Málaga, E-29760 Algarrobo-Costa, Málaga, Spain

[†] Corresponding author: Patricia Bedinger, email bedinger@colostate.edu
* The first two authors contributed equally to the study.

Abstract

Although self-incompatibility (SI) in plants has been studied extensively, far less is known about interspecific reproductive barriers. One interspecific barrier, known as unilateral incongruity or incompatibility (UI), occurs when species display unidirectional compatibility in interspecific crosses. In the wild tomato species *Solanum pennellii*, both SI and self-compatible (SC) populations express UI when crossed with domesticated tomato, offering a useful model system to dissect the molecular mechanisms involved in reproductive barriers. In this study, the timing of reproductive barrier establishment during pistil development was determined in SI and SC accessions of *S. pennellii* using a semi-*in vivo* system to track pollen-tube growth in developing styles. Both SI and UI barriers were absent in styles 5 days prior to flower opening, but were established by 2 days before flower opening, with partial barriers detected during a transition period 3–4 days before flower opening. The developmental expression dynamics of known SI factors, S-RNases and HT proteins, was also examined. The accumulation of HT-A protein coincided temporally and spatially with UI barriers in developing pistils. Proteomic analysis of stigma/styles from key developmental stages showed a switch in protein profiles from cell-division-associated proteins in immature stigma/styles to a set of proteins in mature stigma/styles that included S-RNases, HT-A protein and proteins associated with cell-wall loosening and defense responses, which could be involved in pollen–pistil interactions. Other prominent proteins in mature stigma/styles were those involved in lipid metabolism, consistent with the accumulation of lipid-rich material during pistil maturation.

Key words: pistil development, reproductive barriers, self-incompatibility, *Solanum pennellii*, stigma/stylar proteins, unilateral incongruity/incompatibility.

Introduction

Flowering plants rely predominantly on generalist insect pollinators or other non-specific vectors, such as wind, for the transfer of pollen from one individual to another (Faegri and van der Pijl, 1979; Waser and Ollerton, 2006). Consequently,

they have evolved both prezygotic and postzygotic reproductive barriers to prevent undesirable genomic unions. Prezygotic barriers may contribute more than other mechanisms to outcrossing or reproductive isolation in plants (Rieseberg and Willis, 2007; Lowry *et al.*, 2008). In the Lycopersicon section of *Solanum*, prepollination prezygotic barriers can limit the transfer of pollen from one species to stigmas of other species by mechanisms such as pollinator preference and floral morphology traits (Rick *et al.*, 1978), while postpollination prezygotic barriers act after pollination but before fertilization.

Self-incompatibility (SI) is a postpollination prezygotic intraspecific barrier, wherein self pollen is actively rejected by stigmas or styles to prevent inbreeding depression and facilitate outcrossing. SI systems are well studied and these studies have provided many insights into the mechanisms that prevent self-pollination (e.g., Stephenson *et al.*, 2000; McCubbin and Kao, 2000; Kachroo *et al.*, 2002; Franklin-Tong, 2008; McClure, 2009; Castric *et al.*, 2010). In S-RNase-dependent gametophytic SI prevalent in the Solanaceae, cytotoxic S-RNases and the asparagine-rich HT proteins in styles play key roles in pollen rejection (McClure *et al.*, 1999; O'Brien *et al.*, 2002; McClure and Franklin-Tong, 2006). Unilateral incongruity or incompatibility (UI) is an interspecific barrier that results in rejection of genetically distant pollen genotypes and thereby prevents genomic unions that may undermine fitness. As the name indicates, pollen rejection in UI is one-sided. In most cases, pollen tubes of SI species reach the ovary of SC species, while in reciprocal crosses, pollen tubes of SC species stop growing prior to reaching the ovary of SI species (Lewis and Crowe, 1958). This general trend, called the SI × SC rule, as well as results from genetic and molecular/transgenic analysis, led to the suggestion that UI may simply be an elaboration of SI (Pandey, 1981; Chetelat and DeVerna, 1991; Murfett *et al.*, 1996). However, the role of S-RNases in UI is complex, in that there appear to be both S-RNase-dependent and S-RNase-independent mechanisms for interspecific pollen rejection (Murfett *et al.*, 1996, Covey *et al.*, 2010). In *Nicotiana*, where only a single HT protein is known, this protein is implicated in UI (Hancock *et al.*, 2005). *Solanum* species have at least two highly similar paralogous genes, *HT-A* and *HT-B* (O'Brien *et al.*, 2002; Covey *et al.*, 2010). In the tomato clade, these genes are tandemly duplicated at a locus on chromosome 12 that is linked to a UI quantitative trait locus (QTL; Bernacchi and Tanksley, 1997; Covey *et al.*, 2010). It should be noted, however, there are many exceptions to the SI × SC rule, where SC species, or SC populations within a predominantly SI species, exhibit interspecific pollen rejection (Camadro and Peloquin, 1981; Liedl *et al.*, 1996; Covey *et al.*, 2010) or SI taxa accept pollen from SC relatives (de Nettancourt, 2001). Previous work showed that pollen tube growth differed in SI and UI in *Solanum pennellii*, and that a SC accession of *S. pennellii* could still reject interspecific pollen tubes (Liedl *et al.*, 1996). As early as the 1960s, Grun and Aubertin (1966) surmised from their analysis of UI in *Solanum* species that genes other than SI alleles may contribute to UI. Thus, the relationship between SI and UI remains unclear based on genetic data (Chetelat and DeVerna, 1991; Bernacchi and Tanksley, 1997; Li *et al.*, 2010 and references

therein). Moreover, there are no compelling data indicating that all instances of UI are mechanistically related, and evidence suggests that multiple mechanisms can contribute to UI even between a single pair of species (Murfett *et al.*, 1996).

We have initiated cellular and molecular characterization of interspecific prezygotic mating barriers in the tomato clade (Bedinger *et al.*, 2011; Covey *et al.*, 2010). Section Lycopersicon of the genus *Solanum* is particularly well suited for this analysis. It is a relatively small monophyletic clade consisting of 13 closely related diploid species (domesticated tomato *Solanum lycopersicum* and 12 wild species) with variable mating systems both within and between species (Spooner *et al.*, 2005; Bedinger *et al.*, 2011).

Here, we examined the developmental dynamics of UI and SI in pistils in three accessions of *Solanum pennellii*. In addition, developmental profiles of proteins implicated in SI (S-RNases and the asparagine-rich HT proteins) were assessed using immunological analyses. Furthermore, proteomic profiling of stigma/styles from pre-barrier and post-barrier developmental stages [i.e., 5 days (−5) and 1 day (−1) before flower opening, respectively], validated our immunological analyses of SI factors in developing pistils, and identified a substantial population of proteins present in immature and mature stigma/styles.

Materials and methods

Plant material and growth conditions

One SC (LA0716) and two SI (LA2560 and LA1340) accessions of *S. pennellii* were selected for study. Domesticated tomato *S. lycopersicum* (cv. VF36 or M82) was used as the pollen donor in UI crosses. Plants were grown in Fort Collins, CO, USA, in either in the field (summer 2007) or in the greenhouse (2007–2010) in 25 cm pots filled with Pro-Mix BX, watered regularly and supplemented weekly with Peter's 10-20-10 fertilizer. Natural day-length conditions in the greenhouse were supplemented to a 16h photoperiod. Average temperatures ranged from 27 to 32 °C during the day and were °21 °C at night. In the field, pollinators were excluded by bagging emasculated flowers.

For proteomic analysis, plants were grown both in Colorado as described above and in greenhouses in Ithaca, NY, USA, in order to generate sufficient material. No phenotypic differences were observed between the plants grown in the two locations. Stigma/styles collected in both locations were pooled to minimize the biological variation related to the different locations. Unpollinated stigma/styles from *S. pennellii* SI LA2560 at two different developmental stages (1 and 5 days before the onset of anthesis; termed stages −1 and −5, respectively) were collected and frozen at −80 °C until processing.

Chemicals

All chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St Louis, MO, USA).

Semi-*in vivo* pollinations and style measurements

A semi-*in vivo* system was developed to monitor pollen-tube growth in excised pistils laid on a solid medium [4% polyethylene glycol 8000, 1.6 mM boric acid, 4% sucrose, 3 mM Ca(NO$_3$)$_2$.4H$_2$O, 0.8 mM MgSO$_4$.7H$_2$O, 1 mM KNO$_3$, 20 mM MES, pH 6.0 and 2% agarose] in a petri dish (Fig. 1B). Inflorescences with developing flower buds at stages −1 to −5 (days before the onset of anthesis) were used for pollinations (*n*=48 inflorescences from a minimum of 3–5 plants per accession). Petals and sepals were removed, flowers were emasculated

and stigmas were pollinated with appropriate genotypes. Pistils were placed on the medium immediately (within seconds) after their stigmas were dipped in pollen. Additionally, a paint brush dipped in pollen was used to ensure that stigmas of all pistils in the plate were loaded with pollen. Plates were incubated in a humid chamber at 22 ± 2 °C for 24 h. Sterilization of tissue was deemed unnecessary since no significant microbial growth was observed within this time period. Pollinated pistils were collected after 24 h and fixed, cleared and stained using Aniline Blue fluorochrome {ABF: 4',4-[carbonyl bis(benzene-4,1-diyl)-bis(imino)]-bisbenzensulphonic acid; Biosupplies, Bundoora, VIC, Australia} in 0.1 M K_2PO_4 as a stain for callose in pollen-tube walls (Kearns and Inouye, 1993) as described in Covey *et al.* (2010). Slide-mounted pistils were examined using a Leica DM 5500 B microscope (Leica Microsystems, Buffalo Grove, IL, USA) running IPLab version 4 software (BD Biosciences, San Jose, CA, USA) coupled with a C4742-95 camera (Hamamatsu Photonics, Hamamatsu-City, Japan). Images were captured at 100× magnification using a DAPI filter cube (425 nm long pass; Leica Microsystems). For each pistil (SI n=61; UI n=136), style length was measured from the base of the style to the top of the stigma surface. In each style, the 10 longest pollen tubes were measured from the end of the pollen tube to the top of the stigma and a mean was calculated for use in our analyses (*e.g.* mean pollen-tube length). When pollen tubes reached the ovary, their length was considered equal to the style length. In a few cases fewer than 10 pollen tubes could be measured, in which case we calculated the mean of the measureable tubes. All style and pollen-tube lengths were measured using the programme MicroMeasure version 3.3 (http://rydberg.biology.colostate.edu/MicroMeasure).

We compared mean style length and mean pollen-tube length at five bud stages in SI and UI pollinations with four separate analyses of variance (ANOVAs). For each model we included bud stage (−5 to −1), accession ID (SI, LA2650 and LA1340; UI, LA2650, LA1340 and LA0716) and the interaction term as main effects using the aov (ANOVA) function in the R statistical package (R Development Core Team, 2010). In all four models, there was no significant interaction between bud stage and accession ID, thus the interaction term was dropped. *Post hoc* comparisons of means were made using Tukey's honestly significant difference (HSD; R Development Core Team, 2010).

On-plant pollinations

Pollinations were carried out with flowers still on the plant (UI *n*=18 pollinations; SI *n*=40 pollinations). Unlike the procedure used for semi-*in vivo* studies, only anthers were removed, leaving sepals and petals intact on the flower in order to limit desiccation and mechanical damage. Trilinolein (50 mg), a C_{18} lipid known to promote pollen germination (Wolters-Arts *et al.*, 1998), was emulsified in 0.5 ml of 14% sucrose and 0.01% boric acid (±3 mM calcium nitrate) and used to compensate for the lack of exudate on stigmas of young pistils. Stigmas were painted with this emulsion using a camel-hair brush before applying appropriate pollen samples. Pistils were collected after 24 h and fixed immediately for subsequent processing and staining as described in Covey *et al.* (2010).

Microscopy of pistil tissue

To examine pistil structure at different developmental stages, developmental series of SI LA2560 pistils were fixed in glutaraldehyde, post-fixed and stained with osmium tetroxide, dehydrated with ethanol and embedded in Eponate 12 (Ted Pella, Reading, CA, USA). Sections of 3 μm thickness were cut using an ultramicrotome with a diamond knife. Sections presented in the manuscript were from the midsagittal plane of the stigmas. No additional stains were used and images were collected using a Leica DM5500 B microscope.

Immunoblot analysis

Stylar proteins were extracted and used in immunoblot detection of S-RNase Pen-1, HT-A and -HT-B, as described in Covey *et al.* (2010). In brief, stigma/styles were homogenized in 2 × sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (SDS/PAGE) sample buffer, boiled for 5 min and centrifuged (10 min, 14 000 g). Proteins were separated in a 15% Tris/Tricine gel, blotted onto polyvinylidene difluoride (PVDF) membranes and immunostained. Rabbit Anti-S_{pen-1}-RNase antibody was raised against the synthetic peptide Ac-CTPSKTNTVIDVEKAIKAV-amide, found in S_{pen-1}-RNase from SI *S. pennellii* LA2560 (Covey *et al.*, 2010). The affinity-purified anti-peptide antibody was used at 1:10 000 dilution. HT proteins were extracted and detected as described in Covey *et al.* (2010).

For the immunoblot in Fig. 6, proteins were separated in a 12.5% Tris/Tricine gel, blotted onto PVDF membranes and immunostained with rabbit anti-S-RNase C2 domain antibody. This antibody was raised against the synthetic peptide Ac-NFTIHGLWPD-amide that corresponds to the conserved C2 domain of S-RNases (Haring *et al.*, 1990), and the antibody was affinity purified and used at 1:5000 dilution.

Proteomic analysis

Comparative isobaric tag for relative and absolute quantitation (iTRAQ) proteomic analyses of unpollinated stigma/styles from *S. pennellii* LA2560 at the −5 (UI and SI barriers absent) and −1 (UI and SI barriers present) developmental stages was conducted to assess molecular changes during pistil maturation. iTRAQ is a gel-free comparative proteomic platform that allows the simultaneous identification and relative quantitation of proteins from multiple samples based on the use of unique isobaric tags that identify the sample from which a protein originated (Wise *et al.*, 2007). Proteins were extracted from three equal aliquots of pooled stigma/styles as previously described (Covey *et al.*, 2010) and then reduced, alkylated, proteolytically digested with trypsin and labelled with iTRAQ-4plex reagents, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA; www.applied-biosystems.com/). After labelling, samples were then fractionated by strong cation exchange (SCX) chromatography using an Agilent 1100 HPLC with a UV detector (Agilent Technologies, Santa Clara, CA, USA). The tryptic peptides were dissolved in buffer A (10 mM potassium phosphate, pH 3.0, 25% acetonitrile), prior to SCX separation using a PolyLC PolySulfoethyl A column (2.1 mm × 150 mm; PolyLC, Columbia, MD, USA) eluted with 10 mM potassium phosphate (pH 3.0), 25% acetonitrile with a 0–500 mM KCl gradient. During this elution 40 fractions were collected at a flow rate of 200 μl/min. These were then pooled to create 20 uniformly complex second-dimension fractions based on the UV trace at 214 nm. After solid-phase extraction (Waters SepPak C_{18} cartridge; 1 ml 75% acetonitrile eluent) each fraction was then further fractionated by nanoscale reverse-phase chromatography on an LC Packings UltiMate integrated capillary HPLC with a Dionex (Dionex, Sunnyvale, CA, USA) Switchos valve switching unit and a PepMap C_{18} RP nanocolumn coupled to an LTQ Orbitrap Velos hybrid FT mass spectrometer system (Thermo Scientific, Rockford, IL, USA). This system was used for peptide identification with quality parameters including a 95% confidence index (CI) cutoff, a false discovery rate of 0.05, a *P* value of 0.01 and at least two peptides per protein. Protein extraction and iTRAQ analysis was performed three times with equal aliquots of pooled stigma/styles at each developmental stage. For each of the three iTRAQ experiments about 100 stigma/styles at the −1 stage and about 200 stigma/styles at the −5 stage were used, and extracts were divided into two replicates for each of the three experiments. Those proteins showing more than a 2-fold difference in abundance in at least two iTRAQ experiments were considered to be differentially expressed. The resulting mass spectra data from the iTRAQ runs, corresponding to the different peptides, were analysed using Mascot 2.2 (www.matrixscience.com/) with a protein database built using transcriptomic data derived from 454-based cDNA sequencing (RNA-Seq) from several tomato tissues (stigma/styles, pollen, leaves and fruit; http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/experiment.cgi?ID=D002) together with a customized in-house database

comprising S-RNase sequences annotated in GenBank. Proteomic data can be accessed at www.irbtomato.org. Protein identity was determined using both GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the tomato genome sequence (The Tomato Genome Consortium, 2012).

   N-terminal signal peptide predictions were made using SignalP 4.0 software (www.cbs.dtu.dk/services/SignalP/). For these analyses, predicted protein sequences were from the best matched, most complete codon sequences in GenBank (Supplementary Tables S1 and S2).

Results

Mating systems and developmental staging of S. pennellii

S. pennellii, a member of section Lycopersicon – the tomato clade – has both SI populations, represented here by accessions LA2560 and LA1340, and SC populations, represented by accession LA0716. *S. pennellii*, therefore, provides a useful platform to determine the interrelations and molecular mechanisms regulating both SI and UI reproductive barriers. All accessions of *S. pennellii* used in this study are interfertile and all display UI, in that they reject pollen from SC cultivated tomato, *S. lycopersicum*, while the reciprocal pollination is compatible (Covey *et al.*, 2010).

   Our observations of both SI LA2560 and SI LA1340 show a consistent pre-anthesis floral developmental pattern with buds of successive nodes on a single inflorescence being one day apart in development (Fig. 1A). The node with a freshly opened flower is considered as stage 0 and the subtending nodes are successively staged as −1, −2, −3, etc. In SC accession LA0716, petals begin to separate earlier in bud development, so in this accession stage 0 was assigned to flowers when petals (and anthers) attain full bright yellow color and were completely open. Gradziel and Robinson (1989) also used nodal position as an indicator of bud development in *Solanum peruvianum* but did not correlate this with the chronological age of the buds. Style length of fresh pistils was measured at each stage (−5 to −1) for each accession (Supplementary Fig. S1). As expected, all three accessions showed an increase in style length with bud stage (age), and the growth rate as determined by the slope of the relationship between developmental stage and style length ranged between 1.1 and 1.3 mm/day for the three different accessions throughout the 5-day developmental period under study.

Temporal and spatial onset of reproductive barriers in late pistil development

Using a semi-*in vivo* approach to facilitate pollen adherence and germination at early bud stages (Fig. 1B), we tracked the appearance of SI and UI reproductive barriers during pistil development by pollinating pistils at different developmental stages and observing pollen tubes using ABF staining in fixed pistils (Fig. 2A and 2B). A limited number of on-plant pollinations were performed to confirm the findings with the semi-*in vivo* system (Fig. 2C and 2D). Trilinolein, a major lipid component of stigma exudates in Solanaceae (Matsuzaki *et al.*, 1983; Wolters-Arts *et al.*, 1998), was applied to stigmas
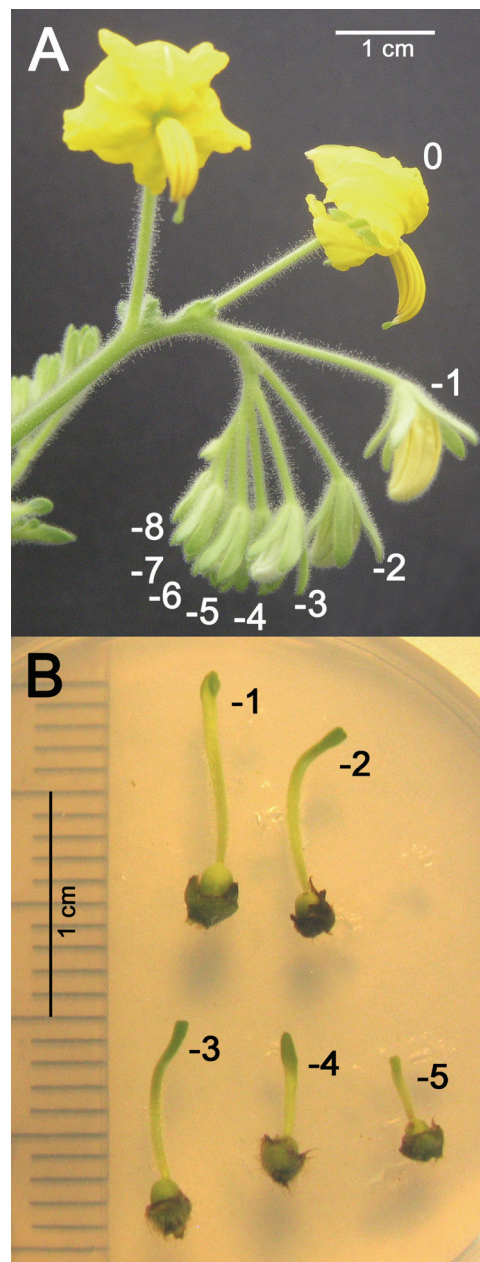

Fig. 1. Developmental staging and semi-*in vivo* pollination system. (A) Developmental staging of flower buds in an inflorescence of *S. pennellii* SI LA2560. Bud break or stage 0 indicates a flower on the first day of its opening. Successive buds are chronologically separated from each other by one day. (B) Pollinated pistils incubated on the medium used in the semi-*in vivo* assay for pollen-tube growth.

of −3 or younger stages to improve pollen retention and germination. However, even with trilinolein application many on-plant pollinations of young pistils demonstrated highly variable pollen adhesion, germination and pollen-tube growth, highlighting the value of a reliable semi-*in vivo* assay for developmental analyses.

   In both SI and UI pollinations, successfully germinated pollen tubes were found to reach the ovary at early bud stages
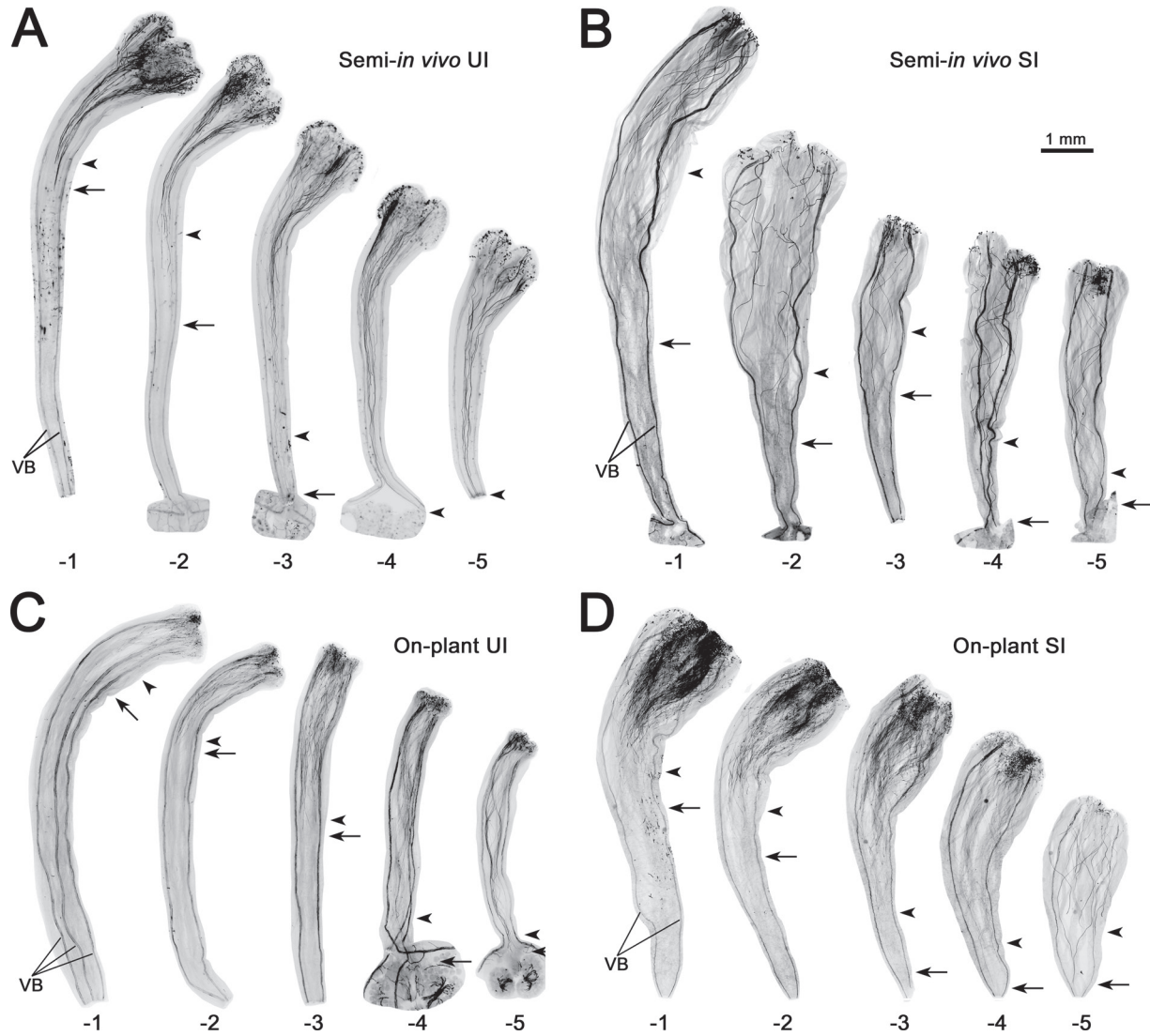
Fig. 2. Pollen-tube growth in UI and SI pollinations. (A) *S. pennellii* accession SI LA2560 pollinated with *S. lycopersicum* cultivar M82 pollen (UI) in the semi-*in vivo* system. (B) *S. pennellii* accession SI LA1340 self pollinated (SI) in the semi-*in vivo* system. (C) *S. pennellii* accession SC LA0716 pollinated by *S. lycopersicum* cultivar M82 (UI) on-plant. (D) *S. pennellii* accession SI LA1340 self-pollinated (SI) on-plant. All pistils were stained with ABF. Arrowheads indicate the point at which most pollen tubes have stopped growing, and arrows indicate the position of the longest pollen tube. VB, vascular bundles.

(−5, −4, −3), and failed to reach the ovary at later bud stages (−1, −2) in the semi-*in vivo* system, suggesting that reproductive barriers are not fully in place 3–5 days before flowering (Fig. 3A and 3B). A similar pattern of pollen-tube growth was found in on-plant UI and SI pollinations, with the exception of bud stage −3, which is likely a transition period for the onset of reproductive barriers (Fig. 3C and 3D). In compatible pollinations (SC LA0716 self-pollination and SI LA2560 or LA1340 sib pollination), pollen tubes reached the ovary at all developmental stages (data not shown).

In a more detailed analysis of pollen-tube length relative to style length, no differences were found among accessions in either style length or mean pollen-tube length by bud stage (Table 1). In UI crosses, mean pollen-tube length (calculated as the mean of the 10 longest pollen tubes/pistil) was significantly shorter at stages −1 and −2 compared to earlier stages

(Fig. 4A, Table 1), suggesting complete onset of UI barriers by bud stage −2. Style length was significantly longer in later stages compared to earlier stages, but this is not likely to be an important factor contributing to reproductive isolation in these UI crosses, because the net length reached by pollen tubes in UI rejection is less than the length of a −5 stage style.

In SI pollinations, mean pollen-tube length was more variable than in UI crosses, and the timing of the onset of SI reproductive barriers was not reflected in an abrupt change in mean pollen-tube length (Fig. 4B, Table 1). As in the UI crosses, style length was significantly longer in later stages (−1 and −2) relative to early stages (Fig. 4B, Table 1). In SI, style length in conjunction with the known developmental regulation of SI factors (McClure *et al.*, 1999 and see below) probably contributes to the onset of SI reproductive barriers.
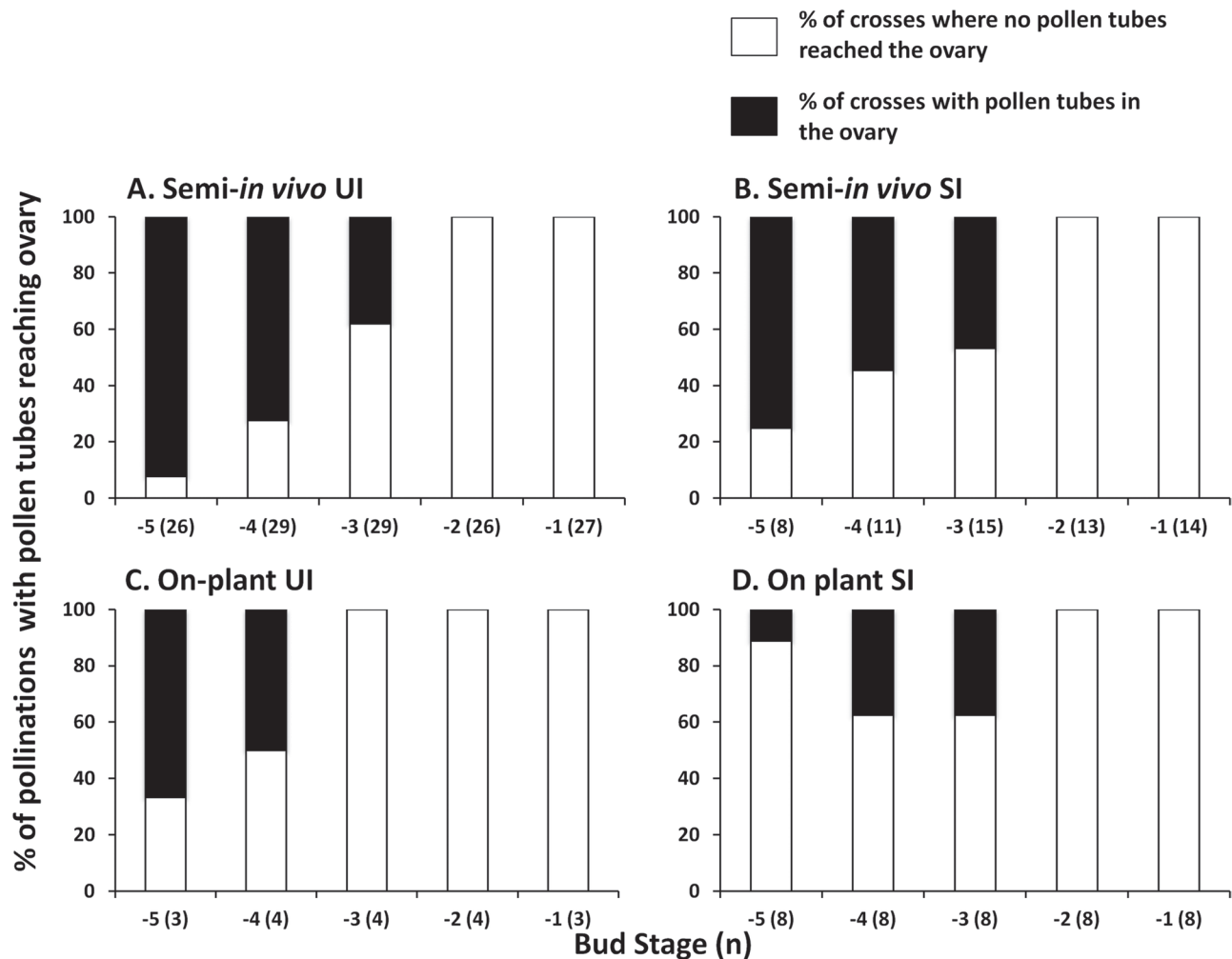
Fig. 3. Pollen tubes at ovaries in UI and SI pollinations of developmentally staged pistils. The percentage of pollinations that resulted in pollen tubes reaching the ovary (black) or with no pollen tubes reaching the ovary (white) at five bud stages in semi-*in vivo* UI (A) and SI (B) pollinations and in on-plant UI (C) and SI (D) pollinations. The number of images analysed for each type of pollination is shown in parentheses.

The location in the style of reproductive isolating barriers differed between UI and SI, as has previously been reported (Hardon, 1967; Gradziel and Robinson, 1989; Kuboyama *et al*., 1994; Liedl *et al*., 1996; Covey *et al*., 2010) (Fig. 4). At later stages (−1 and −2) in SI pollinations, pollen-tube growth ceased in the mid-region of the style, whereas in UI crosses pollen stopped in the upper third of the style.

SI factor accumulation during pistil development

S-RNases and HT proteins are essential SI factors produced in the style. We prepared antibodies specific for HT-A, HT-B and an S-RNase previously identified in LA2560, Pen-1 (Covey *et al*., 2010), to enable comparison of the expression profile of these proteins with the development of pollination barriers (Fig. 5). Our results show that considerable S-RNase protein was present in stigma/styles of SI *S. pennellii* LA2560 at stage −4 (Fig. 5A), suggesting that S-RNase alone is not sufficient for full rejection of either self or interspecific pollen, since

barriers are not fully established until stage −2 (Figs 2–4). In fact, S-RNases are not detected in the SC accession LA0716 (Fig. 6), which exhibits UI pollen-tube rejection as rapidly as the SI accessions (Liedl *et al*., 1996; Covey *et al*., 2010). In both SI and SC *S. pennellii* accessions, HT-A protein was detected from stage −3 onwards, while HT-B was not detectable until stage −1 (Fig. 5). Although proteomic analysis (presented below) confirms the low abundance of HT-B relative to HT-A, the apparent temporal difference in HT-A and HT-B expression seen here could also be attributed to a relatively lower sensitivity of the HT-B antibodies compared to the HT-A antibodies used in this study (*i.e.* trace levels of HT-B present at earlier stages may not have been detected). In summary, both SI and UI pollen rejection commences by stage −2, when significant levels of both HT-A and S-RNase proteins were detected.

Fig. 7 compares HT-A protein accumulation in upper and lower portions of the stigma/style in *S. pennellii* accessions SI LA2560 and SC LA0716. The higher abundance of HT protein in the upper half of the stigma/style corresponds to the
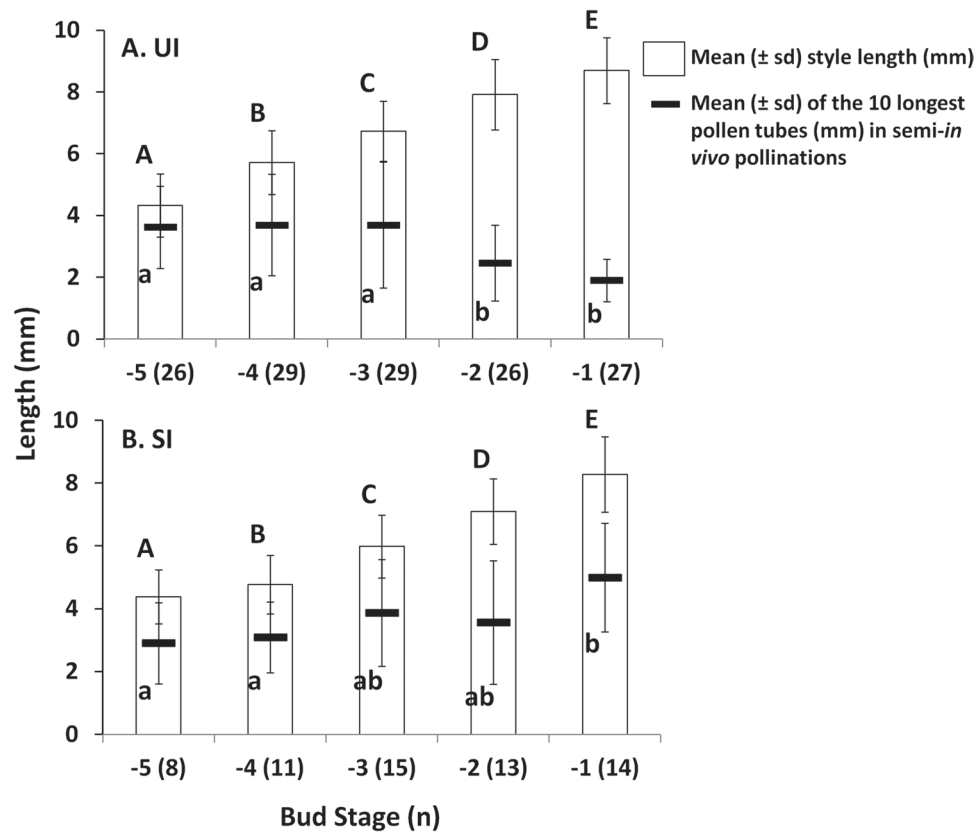
Fig. 4. Analysis of pollen-tube growth in UI and SI in semi-*in vivo* pollinations of developmentally staged pistils. (A) The mean (±sd) style length (white bars) and the grand mean (±sd) of the 10 longest pollen tubes (black horizontal line) in UI crosses of *S. pennellii* with cultivated tomato. (B) The mean (±sd) style length (white bars) and the grand mean (±sd) of the 10 longest pollen tubes (black horizontal line) in SI pollinations of *S. pennellii* with self pollen. Significant differences (*P*<0.05) within each pollination type (SI and UI) for style length and for mean pollen-tube length based on Tukey's (honestly significant difference) *post hoc* tests are denoted within each figure by upper-case letters for style length and lower-case letters for mean pollen-tube length. The number of images analysed for each type of pollination is shown in parentheses.

site of UI pollen rejection. It should be noted that although expression of HT-A (in the upper stigma/style) is detected in *S. lycopersicum* (Fig. 7, arrowhead), this truncated HT-A lacks the asparagine-rich domain and thus is presumably non-functional in this fully compatible species (Kondo *et al.*, 2002*a*; Covey *et al.*, 2010).

Proteomic analysis of stigma/styles from key developmental stages

iTRAQ profiling of −1 and −5 stigma/styles of SI accession LA2560 resulted in the identification and quantification of a total of 2534 stylar proteins with a 95% CI from three

Table 1. Results from four separate ANOVAs examining the effects of accession ID and bud stage on mean pollen-tube length and mean style length in SI and UI crosses.

Response variable	Pollination type	Factor	df	*F* value	*P* value
Mean pollen-tube length	UI	Accession ID	2	0.077	0.93
		Bud stage	4	8.78	<0.000001*
	SI	Accession ID	2	1.04	0.31
		Bud stage	4	2.92	0.03*
Mean style length	UI	Accession ID	2	1.72	0.18
		Bud stage	4	72.84	<0.000001*
	SI	Accession ID	2	0.81	0.37
		Bud stage	4	29.44	<0.000001*

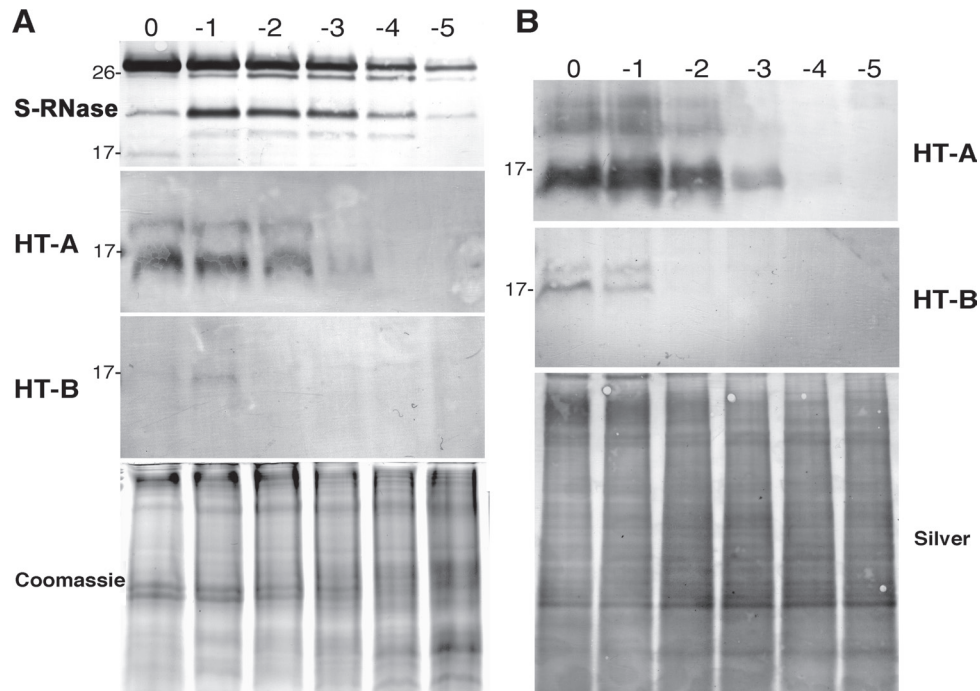*Results of pairwise *post hoc* tests are shown in Fig. 4.

Fig. 5. SI proteins during pistil development in *S. pennellii.* (A) *S. pennellii* SI LA2560; (B) *S. pennellii* SC LA0716. Immunoblots (1.2 mg fresh weight/lane) show relative abundance and time of expression of S-RNase (S$_{pen-1}$-RNase, an S-RNase previously identified in LA2560), HT-A and HT-B proteins in developing pistils. Protein sizes (kDa) are given on the left. Bottom panels show Coomassie- or silver-stained gels of the pistil extracts as loading controls.
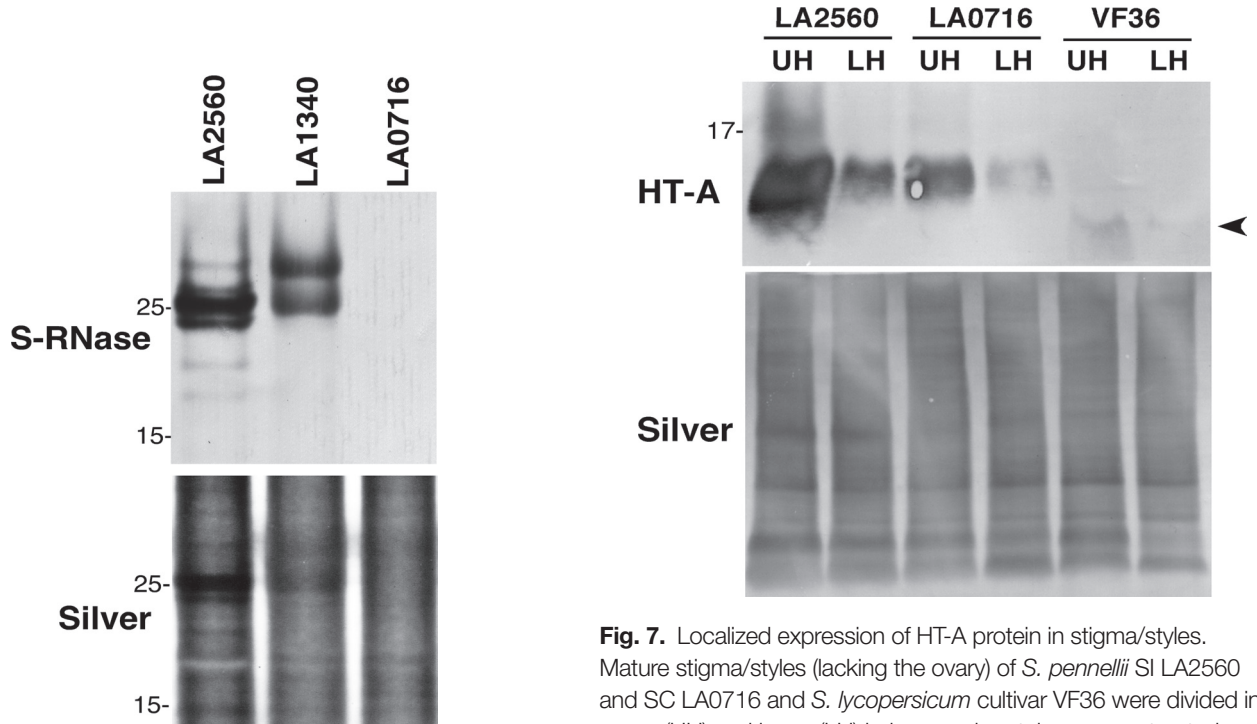

Fig. 6. Relative levels of S-RNases in *S. pennellii* accessions SI LA2560, SI LA1340 and SC LA0716. S-RNases in protein extracts (0.2 mg fresh weight/lane) from mature pistils were immunostained with anti-S-RNase C2 domain antibody. Silver staining of the blotted protein extracts acted as a loading control.

Fig. 7. Localized expression of HT-A protein in stigma/styles. Mature stigma/styles (lacking the ovary) of *S. pennellii* SI LA2560 and SC LA0716 and *S. lycopersicum* cultivar VF36 were divided into upper (UH) and lower (LH) halves, and proteins were extracted and separated on SDS/PAGE gels. Upper panel: immunostaining of pistil extracts using anti-HT-A antibody (1.2 mg fresh weight/lane). Protein size (kDa) is given on the left. Arrowhead on the right indicates the predicted truncated form of HT-A in cultivar VF36. Silver staining of the blotted protein extracts acted as a loading control.
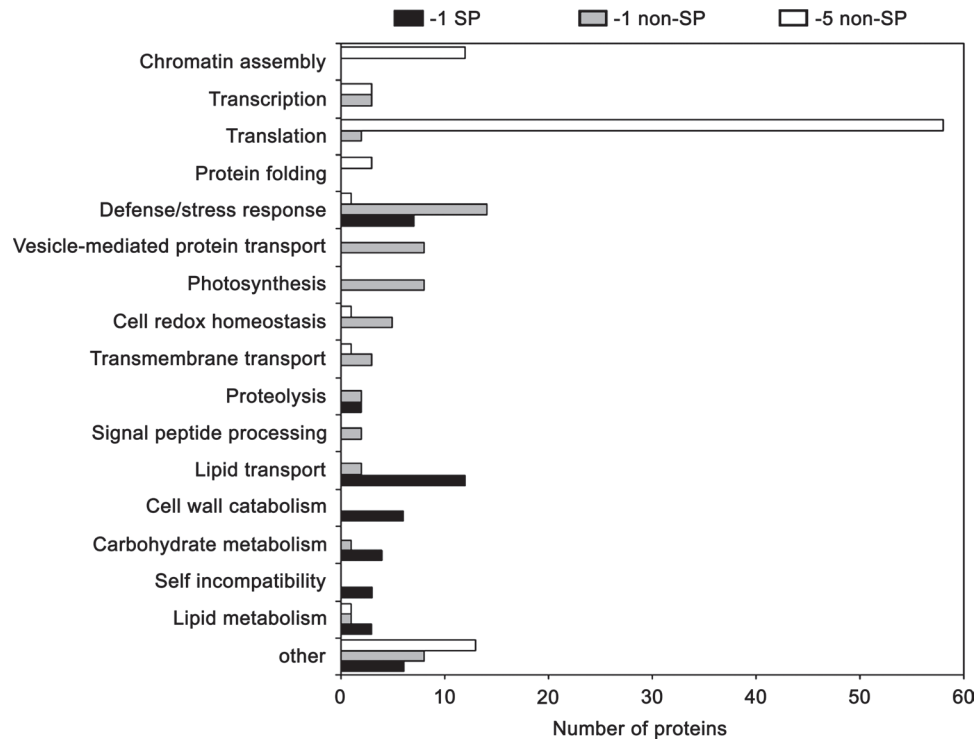
Fig. 8. Biological process ontology classification of the top 100 differentially expressed proteins in inmmature (−5 stage) and in mature (−1 stage) stigma/styles. Proteins were categorized as having a signal peptide (SP) or not having a signal peptide (non-SP) based on N-terminal signal peptide prediction analysis using SignalP 4. Only three −5 SP proteins were identified and are not shown. Classification is according to the AmiGO gene ontology database (http://amigo.geneontology.org).

separate iTRAQ experiments. From this group, 2015 proteins were identified with more than one peptide, allowing relative quantification within each sample. For determination of the relative abundance of the proteins in each sample type, a selection parameter requiring the detection of any identified protein in at least two of the three iTRAQ experiments was applied. A total of 1312 proteins met all three conditions (95% CI, more than one peptide detected, and detected in at least two of three experiments) and were used in quantitative analysis. Of these, 498 were found to be more abundant in stage −5, whereas 131 were more abundant in stage −1, based on a cutoff of 2-fold or greater difference. A summary of functional annotations of these proteins is shown in Fig. 8.

The top 100 proteins that showed the greatest relative abundance at the −5 stage (immature stigma/styles) are shown in Supplementary Table S1. Prominent proteins in this group include histones (11%) and ribosomal proteins (58%), consistent with a phase of cell division and elevated cellular metabolism in immature stigma/styles. The top 100 proteins with a higher relative abundance at the −1 stage (mature stigma/styles) are listed in Supplementary Table S2. This group included proteins associated with lipid metabolism and transport, with 14% annotated as lipid transfer proteins. Accumulation of lipids is a hallmark of pistil maturation in most plant species with wet stigmas, including those in Solanaceae (Konar and Linskens, 1966; Dumas *et al.*, 1978; Matsuzaki *et al.*, 1983; Nakamura *et al.*, 2003, 2009), Stigma/styles at the −5 stage lacked a fully differentiated transmitting

tract with its distinctive elongated cells, and also had very little or no lipid secreted onto the stigma surface, as determined from observations of sections of osmium tetroxide-stained stigma/styles (Fig. 9). In −1 and −2 stage stigma/styles, lipids are a prominent feature both on the stigma surface and between the cells of the now recognizable transmitting tissue (Fig. 9). These observations are consistent with the proteomics results and with the poor retention and growth of pollen on stigmas of stages younger than −3 in on-plant pollinations. Other proteins that were prominent at the −1 stage include arabinogalactan proteins, defensin, thaumatin, chitinase, HT-A proteins and S-RNases, among others (Supplementary Table S2). We performed a search for predicted N-terminal secretion signal peptides (SPs) using SignalP 4 with sequences of the most differentially expressed proteins at the −5 and −1 developmental stages (SP; Supplementary Tables S1 and S2, Fig. 8) using the most complete codons available for those sequences with a match in GenBank (www.ncbi.nlm.nih.gov/genbank/; 96/100 for the −5 stage and 95/100 for the −1 stage). SP prediction analysis of differentially expressed proteins in the two stages revealed a major shift towards a secretory function in mature stigma/styles. Only 3% of the top 100 proteins at the −5 stage stigma/styles were predicted to contain a SP, compared with 38% of proteins at the −1 stage.

Known SI proteins were quantified in SI LA2560 stigma/styles at the −1 and −5 stages. HT-A proteins were detected at higher levels at the −1 stage relative to the −5 stage, consistent with immunoblotting results (Fig. 5), and were represented
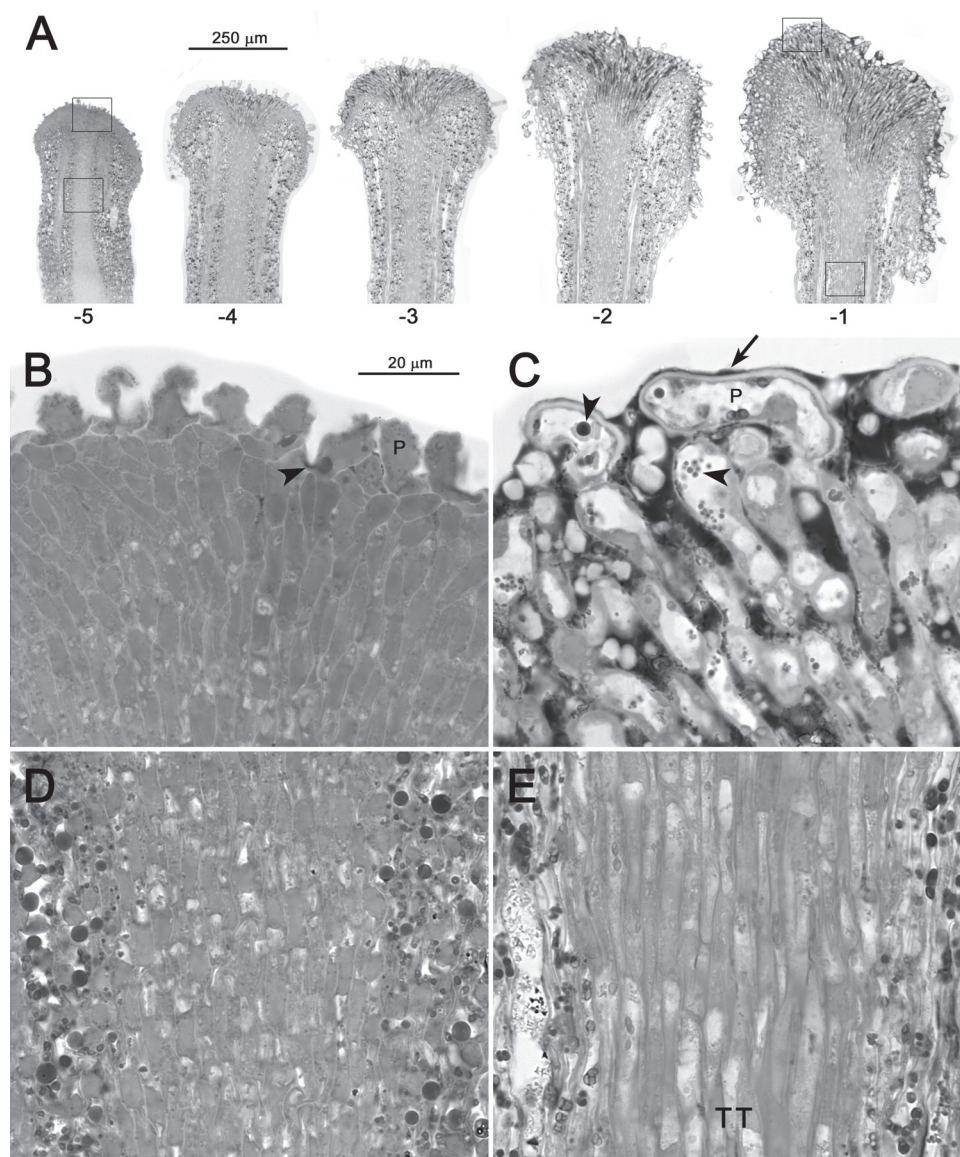
Fig. 9. Stigma/style development in *S. pennellii*. (A) Longitudinal sections of osmium tetroxide-stained, plastic-embedded pistils of S. *pennellii* SI LA2560 at −5 to −1 days before bud opening. An increase in stigma size and the amount of black, osmiophilic (presumably lipid) material in the stigma/styles is apparent during pistil development. The sections shown were taken from approximately the mid-sagittal planes of the stigmas. (B, C) Enlarged images of sections of the stigmas of −5 (B) and −1 (C) pistils, taken from the areas within the upper boxes in (A). There is little intercellular space among the stigma cells at −5, and only a small amount of osmiophilic material (arrowhead) is present in some cells at the surface. Papillae (P) are minimally developed. By −1, the intercellular space among the cells of the stigma and upper style is greatly increased and filled with osmiophilic material which also covers the surface of papillae in the stigma (arrow). Globules of osmiophilic material are also abundant within papillae and in cells below the stigma surface (arrowheads). Papillae are well developed and have thickened cell walls. (D, E) Enlarged images of sections of stigma/styles at −5 (D) and −1 (E), taken from the regions in the lower boxes in (A). At −5 the cells in the central part of the style are relatively undifferentiated. By −1 the well-developed transmitting tract (TT) consists of markedly elongated cells. At both stages, osmiophilic globules interpreted as lipids are abundant within peripheral cells of the style. Scale bar in (B) also applies to (C–E).

by at least four isoforms (Supplementary Table S3). HT-B protein was not detected in the proteomic analysis, suggesting a lower abundance of HT-B compared to HT-A. In addition to the two S-RNases shown in Supplementary Table S1, peptides corresponding to six additional S-RNases were identified in −1 stigma/styles of LA2560 (Supplementary Table S4), including one previously described in SI LA2560 (Pen-1; Covey *et al.*, 2010). In this group, one S-RNase was identified and quantified using a custom database generated from 454-based cDNA sequencing (RNA-Seq) data (TU156268; see Materials and Methods) and five were identified using a database collected from solanaceous S-RNase sequences annotated in GenBank. Since the latter contains only S-RNase sequences, search restriction parameters were increased to

compensate for potential bias, such that only those proteins identified with at least five different peptides were included in Supplementary Table S4.

Discussion

Developmental control of reproductive barriers

Developmental regulation of reproductive barrier establishment is known to occur, since bud pollinations have been used to successfully overcome both interspecific and selfing barriers in many species (Gradziel and Robinson, 1989, 1991; Kuboyama *et al.*, 1994; Sánchez *et al.*, 2004; Singh *et al.*, 2004). For example, UI barriers between cultivated tomato and either *Solanum lycopersicoides* or *S. peruvianum* were circumvented by bud pollinations using a stigma complementation procedure on otherwise non-receptive stigmas of pre-anthesis buds (Gradziel and Robinson, 1989, 1991). Bud pollinations have also been successful in overcoming reproductive barriers in other Solanaceae members, such as *Nicotiana tabacum* and *Petunia hybrida* (Sink and Power, 1978; Kuboyama *et al.*, 1994; Sánchez *et al.*, 2004).

Here we compare the timing of SI and UI barrier establishment in pistils of three accessions of a single species, and present a detailed pollen-tube growth analysis during pistil development. The predictable sequential bud maturation on *S. pennellii* inflorescences and the development of a robust semi-*in vivo* pollination system (Figs 1A, B and 2) contributed to the success of our studies. The semi-*in vivo* system yielded consistent results by minimizing environmental and endogenous factors known to influence the results of on-plant bud pollinations in many plant systems, including wild species of tomato (Emerson, 1940; Linskens, 1975; William and Knox, 1982; Webb and Williams, 1988 and references therein; Gradziel and Robinson 1989). The variability of these factors can confound results and lead to conflicting inferences. For example, differences in temperature, availability of nutrients and photoperiod can contribute to the strength of SI reactions (Webb and Williams, 1988 and references therein). The semi-*in vivo* pollination system allowed the reliable tracking of both SI and UI reproductive barriers during style development. Both types of barrier are absent 5 days before flower opening, and barriers are fully established by 2 days before flower opening (Figs 3 and 4).

Our results as well as previous reports (e.g., Gradziel and Robinson, 1989, 1991; Kuboyama *et al.*, 1994) show that the pistil becomes competent to support directional pollen-tube growth early during its development, but flower opening is delayed until the pistil establishes the molecular machinery that can discriminate between genetically compatible and incompatible pollen. Since *S. lycopersicum* pollen tubes grew within immature styles successfully to the ovary in *S. pennellii* pistils prior to barrier establishment, our results are more consistent with an active rejection (incompatibility) as the underlying mechanism for interspecific pollen rejection than a mismatch between pollen and pistil components (incongruency), with reproductive barriers layered on top of an otherwise compatible system. However, incongruency remains an explanation for failure of interspecific crosses in some systems (Kermicle and Evans, 2005), and this phenomenon is likely to be widespread (Hogenboom, 1975).

SI-related proteins and the onset of UI

Immunoblotting results (Fig. 5) show that the accumulation of HT-A protein coincides with the manifestation of UI barriers both in SI LA2560 and in SC LA0716. Proteomic analysis confirmed that HT-A protein was differentially expressed in the −1 stage compared to the −5 stage in SI LA2560 (Supplementary Table S1). HT-B was not detected until later; a similar delay in the appearance of *HT-B* over *HT-A* transcripts in the stylar tissues of *Solanum chacoense* was reported by O'Brien *et al.* (2002). Although the HT-B antibody used is highly selective, it has a lower sensitivity than the HT-A antibody. Thus, the relative timing of HT-A versus HT-B expression is less clear than the overall correlation between HT-A expression and UI pollen rejection. Moreover, several lines of evidence point to a role for HT proteins in UI. First, the two tightly linked HT paralogs map to a pistil-side QTL controlling UI in the tomato clade (Bernacchi and Tanksley, 1997; Covey *et al.*, 2010). Second, HT expression patterns are correlated with UI at the species level. For example, all four of the SC red-fruited species (*S. lycopersicum*, *Solanum pimpinellifolium*, *Solanum galapagense* and *Solanum cheesmaniae*) in the tomato clade lack UI barriers and lack both HT-A and HT-B proteins due to mutations in both genes that create early termination codons (Kondo *et al.*, 2002a, 2002b). The two SC green-fruited species (*Solanum neorickii* and *Solanum chmielewskii*) have UI barriers and express HT-A protein (Kondo *et al.*, 2002a). All tested SI accessions in the tomato clade have UI barriers and express both S-RNase and HT-A (Kondo *et al.*, 2002a; Covey *et al.*, 2010). The spatial correlation between UI and HT-A protein accumulation presented here is also consistent with a role for HT proteins in UI (Fig. 7).

Both S-RNase and asparagine-rich HT proteins are known to be required for SI because suppression of either S-RNase or HT protein prevents *S*-allele-specific self pollen rejection (Murfett *et al.*, 1995; McClure *et al.*, 1999; Hancock *et al.*, 2005; Puerta *et al.*, 2009). In addition, some types of UI have been shown to require HT proteins in *Nicotiana* (Hancock *et al.*, 2005). Our results showing significant accumulation of S-RNase prior to the onset of SI or UI in *S. pennellii* SI LA2560 (Fig. 5) are consistent with the regulation of the SI system in *Nicotiana*, where S-RNase transcript accumulation precedes HT transcript accumulation, and SI establishment requires sufficient levels of both proteins (McClure *et al.*, 1999). The difference between HT-protein accumulation in the upper versus lower half of the stigma/style (Fig. 7) correlates with the site of pollen rejection. The greater abundance of HT protein in the upper stigma/style is not likely to be attributable to higher expression in the stigma, since studies in *Nicotiana* showed moderately lower HT-transcript levels in stigma versus style (McClure *et al.*, 1999). These results must not be interpreted to suggest that all active UI mechanisms are S-RNase-dependent. For example, SC *S. pennellii*

LA0716 lacks any detectable S-RNase protein or activity, but displays strong UI pollen rejection (Fig. 6, Liedl *et al.*, 1996; Covey *et al.*, 2010). Thus, redundant S-RNase-dependent and S-RNase-independent mechanisms are likely to contribute to UI in the tomato clade, and the loss of SI (as is the case to SC *S. pennellii* LA0716) does not necessarily lead to a loss of UI.

Changes in the stigma/stylar proteome associated with reproductive barrier establishment

Proteomic analyses of developmentally staged SI LA2560 stigma/styles revealed that the top 100 most abundant proteins at the earlier developmental stage (−5) featured histones and ribosomal proteins, reflecting a high rate of cell division and active metabolism (Supplementary Table S1, Fig. 8). At the later developmental stage (−1), when styles are able to reject incompatible pollen, the most abundant group of proteins is annotated as potentially associated with lipid metabolism [Supplementary Table S2, Fig. 8, e.g. lipid-transfer protein (LTP) and GDSL]. LTPs are proteins with a wide range of biological roles (Yeats and Rose, 2008) and these stylar LTPs may be functionally associated with the accumulation of large amounts of secreted lipid on the stigma and within the transmitting tract of stigma/styles immediately prior to flower opening (Fig. 9). Solanaceous species such as tomato, tobacco and petunia have wet stigmas with large amounts of lipid-rich exudates that peak during anthesis (Konar and Linskens, 1966; Dumas *et al.*, 1978; Herrero and Dickinson, 1980; Matsuzaki *et al.*, 1983; Nakamura *et al.*, 2003, 2009), and these exudates are critical for controlled pollen hydration (Wolters-Arts *et al.*, 1998; Lush, 1999).

Some of these LTPs, as well as other highly abundant proteins detected at this −1 stage, such as defensins and thaumatins, can be classified as cysteine-rich proteins (CRPs). Interestingly, some CRPs seem to have evolved functions in pollen-pistil interactions (Marshall *et al.*, 2011). For example, Stig1 is a stigma-specific CRP involved in exudate formation on stigmas and possibly in promoting pollen-tube growth (Tang *et al.*, 2004; Verhoeven *et al.*, 2005). Pistil LTPs facilitate pollen-tube adhesion and growth in the transmitting tract of lily and *Arabidopsis* styles (Park and Lord, 2003; Tung *et al.*, 2005; Chae *et al.*, 2007, 2009, 2010). CRPs that are more abundant in −1 stage stigma/styles could play roles in defense (e.g. Lin *et al.*, 2007; Hammami *et al.*, 2009), since the stigma/styles at this stage are hydrated, nutrient-rich and exposed to the environment, and therefore accessible to pathogens, or could function in pollen–pistil interactions (Kessler *et al.*, 2010). Defensin-like proteins contribute to a number of reproductive processes, including pollen viability (Stotz *et al.*, 2009), pollen hydration (Muschietti *et al.*, 1994), pollen–stigma recognition in sporophytic SI in *Brassica* (Kachroo *et al.*, 2002), pollen-tube guidance to embryo sacs (Okuda *et al.*, 2009) and the regulated release of sperm cells from pollen tubes (Amien *et al.*, 2010). At least 43% of proteins at the −1 stage have predicted ER-targeting SPs, consistent with the importance of membrane-localized and secreted proteins in pollen-pistil interactions. Among these secreted proteins we noted a number of predicted cell-wall-remodelling proteins,

including pectinases (four distinct putative pectate lyase proteins), and a polygalacturonase. Both of these classes of proteins presumably act to depolymerize the pectins in the primary cell wall and, perhaps more importantly, the middle lamella, a structure that may be less prominent in *Solanum* spp. than in other taxa, although the intercellular material has been suggested to contain pectins (Cresti *et al.*, 1976). In order for pollen tubes to penetrate the stylar tissues and reach the ovary, they need to pass between the transmitting tract cells. We suggest that in mature pistils these pectinases act to degrade the primary wall of the transmitting tract cells, or the pectin component of the intercellular material to facilitate this process, as has been suggested previously for various pectinases (Marín-Rodríguez *et al.*, 2002). A predicted expansin, another class of protein with a well-established role in cell-wall loosening, is also in the top differentially expressed 100 −1 stage proteins. Expansins in pollen have been previously proposed to promote stylar penetration (Wu *et al.*, 2001), and their presence in styles could also promote stylar penetration by pollen tubes.

Proteome characterization of SI LA2560 stigma/styles also confirmed the presence of S-RNases and HT proteins (Supplementary Tables S1, S2 and S3), which are known to contribute to SI in solanaceous species. Relative quantification confirmed that both of these classes of protein were more abundant in −1 stage stigma/styles than in −5 stigma/styles, correlating temporally with pistil maturity and the manifestation of reproductive barriers. Our results therefore provide targets for future studies aimed at elucidating the multiple complex pathways that are UI-associated.

Supplementary material

Supplementary material is available at *JXB* online.

Supplementary Fig. S1. Style length during flower development. Fresh styles of −5, −4, −3, −2 and −1 stage flowers from *S. pennellii* accessions LA2560, LA1340 and LA0716 were imaged immediately after placement of pistils on medium, and measurements were made using the ImageJ 1.33 programme. N, number of images analysed. Error bars indicate standard error of the mean.

Supplementary Table S1. The top 100 differentially expressed proteins in stigma/styles of *S. pennellii* SI LA2560 at the −5 stage (immature stigma/styles). SP indicates N-terminal signal peptide prediction using the SignalP 4 programme; Y and N indicate secreted and non-secreted, respectively, according to the prediction. Ratio −1/−5 indicates the average relative quantification at the −1 stage with respect to the −5 stage. SD is the standard deviation associated with the average ratio. Np indicates the number of peptides used for quantification, with a range indicated when different numbers of peptides were used in different experiments. NR indicates number of iTRAQ experiments in which the protein was identified (out of three possible).

Supplementary Table S2. The top 100 differentially expressed proteins in stigma/styles of *S. pennellii* SI LA2560 at the −1 stage (mature stigma/styles). SP indicates N-terminal

signal peptide prediction using the SignalP 4 programme; Y and N indicate secreted and non-secreted, respectively, according to the prediction. Ratio −1/−5 indicates the average relative quantification at the −1 stage with respect to the −5 stage. SD is the standard deviation associated with the average ratio. Np indicates the number of peptides used for quantification, with a range indicated when different numbers of peptides were used in different experiments. NR indicates number of iTRAQ experiments in which the protein was identified (out of three possible).

Supplementary Table S3. HT-A peptides detected in stigma/styles of *S. pennellii* SI LA2560. Peptide sequences represent HT-A proteins described in Covey *et al.*, 2010, as well as three additional HT-A peptides. Ratio −1/−5 indicates the average relative quantification in the −1 stage relative to the −5 stage. SD means standard deviation associated with the average calculation. NR indicates number of iTRAQ experiments in which the protein was identified (out of three possible). Np indicates the number of peptides used for quantification. NA means that the peptide was not used for quantification. Peptides are shown according to their position in the protein, from N- to C-terminus. The first four peptides are detected in *S. lycopersicum* cv. VF36, but not the remaining five peptides, which are distal to the premature termination site that produces a truncated protein in this species.

Supplementary Table S4. S-RNases identified in stigma/styles of *S. pennellii* SI LA2560. The S-RNases in this table are in addition to the two S-RNases in Supplementary Table S2. −1/−5 indicates the average relative quantification in −1 stage respect to the −5 stage. SD means standard deviation associated with the average calculation. Np indicates the number of peptides used for quantification. NR indicates number of iTRAQ experiments in which the protein was identified (out of three possible).

Acknowledgements

References

Amien S, Kliwer I, Márton ML, Debener T, Geiger D, Becker D, Dresselhaus T. 2010. Defensin-like ZmES4 mediates pollen tube burst in maize via opening of the potassium channel KZM1. *PLOS Biology* **8,** e1000388.

Bedinger PA, Chetelat RT, McClure BA, Moyle LC, Rose JKC, Stack SM, van der Knaap E, Baek YS, Lopez-Casado G, Covey PA, Kumar A, Li W, Nunez R, Cruz-Garcia F, Royer S. 2011. Interspecific reproductive barriers in the tomato clade: opportunities to decipher mechanisms of reproductive isolation. *Sexual Plant Reproduction* **24,** 171–187.

Bernacchi D, Tanksley SD. 1997. An interspecific backcross of *Lycopersicon esculentum* x *L. hirsutum*: linkage analysis and a QTL study of sexual compatibility factors and floral traits. *Genetics* **147,** 861–877.

Camadro EL, Peloquin SJ. 1981. Cross-incompatibility between two sympatric polyploid *Solanum* species. *Theoretical and Applied Genetics* **60,** 65–70.

Castric V, Bechsgaard JS, Grenier S, Noureddine R, Schierup MH, Vekemans X. 2010. Molecular evolution within and between Self-Incompatibility species. *Molecular Biology and Evolution* **27,** 11–20.

Chae K, Li Z, Li K, Morikis D, Kim ST, Mollet JC, de la Rosa N, Tan K, Lord EM. 2007. Two SCA (stigma/style cysteine-rich adhesin) isoforms show structural differences that correlate with their levels of in *vitro* pollen tube adhesion activity. *Journal of Biological Chemistry* **282,** 33845–33858.

Chae K, Kieslich CA, Morikis D, Kim, SC, Lord EM. 2009. A gain-of-function mutation of Arabidopsis lipid transfer protein 5 disturbs pollen tube growth and fertilization. *The Plant Cell* **21,** 3902–3914.

Chae K, Gonong BJ, Kim SC, Kieslich CA, Morikis D, Balasubramanian S, Lord EM. 2010. A multifaceted study of stigma/style cysteine-rich adhesin (SCA)-like Arabidopsis lipid transfer protein (LTPs) suggests diversified roles for these LTPs in plant growth and reproduction. *Journal of Experimental Botany* **61,** 4277–4290.

Chetelat RT, DeVerna JW. 1991. Expression of unilateral incompatibility in pollen of *Lycopersicon pennellii* is determined by major loci on chromosome 1, 6 and 10. *Theoretical Applied Genetics* **82,** 704–712.

Covey PA, Kondo K, Welch L, Frank E, Sianta S, Kumar A, Nuñez R, Lopez-Casado G, Van Der Knaap E, Rose JKC, McClure BA, Bedinger PA. 2010. Multiple features that distinguish unilateral incongruity and self-incompatibility in the tomato clade. *The Plant Journal* **64,** 367–378.

Cresti M, van Went JL, Pacini E, Willemse MTM. 1976. Ultrastructure of transmitting tissue of *Lycopersicon peruvianum* style: development and histochemistry. *Planta* **132,** 305–312.

de Nettancourt D. 2001. *Incompatibility and Incongruity in Wild and Cultivated Plants*. Springer-Verlag, Berlin.

Dumas CM, Rougier M, Zandonella P, Ciampolini F, Cresti M, Pacini E. 1978. The secretory stigma in *Lycopersicon peruvianum* Mill.: ontogenesis and grandular activity. *Protoplasma* **96,** 173–187.

Emerson S. 1940. Growth of incompatible pollen tubes in *Oenothera organensis*. *Botanical Gazette* **101,** 890–911.

Faegri K, van der Pijl L. 1979. *The Principles of Pollination Ecology*, Ed 3. Pergamon, Oxford.

Franklin-Tong V. 2008. *Self-Incompatibility in Flowering Plants – Evolution, Diversity, and Mechanisms*. Springer-Verlag, Berlin.

Gradziel TM, Robinson RW. 1989. Breakdown of self-incompatibility during pistil development in *Lycopersicon peruvianum* by modified bud pollination. *Sexual Plant Reproduction* **2,** 38–42.

Gradziel TM, Robinson RW. 1991. Overcoming unilateral breeding barriers between *Lycopersicon peruvianum* and cultivated tomato, *Lycopersicon esculentum*. *Euphytica* **54,** 1–9.

Grun P, Aubertin M. 1966. The inheritance and expression of unilateral incompatibility in *Solanum*. *Heredity* **21,** 131–138.

Hammami R, Ben Hamida J, Vergoten G, Fliss I. 2009. PhytAMP: a database dedicated to antimicrobial plant peptides. *Nucleic Acids Research* **37,** D963–D968.

Hancock CN, Kent L, McClure BA. 2005. The stylar 120 kDa glycoprotein is required for S-specific pollen rejection in *Nicotiana*. *The Plant Journal* **43,** 716–723.

Hardon JJ. 1967. Unilateral incompatibility between SOLANUM PENNELLII and LYCOPERSICON ESCULENTUM. *Genetics* **57,** 795–808.

Haring V, Gray JE, McClure BA, Anderson MA, Clarke AE. 1990. Self-incompatibility: a self-recognition system in plants. *Science* **250,** 937–941.

Herrero M, Dickinson HG. 1980. Ultrastructural and physiological differences between buds and mature flowers of *Petunia hybrida* prior to and following pollination. *Planta* **148,** 138–145.

Hogenboom NG. 1975. Incompatibility and incongruity: Two different mechanisms for the non-functioning of intimate partner relationships. *Proceedings of The Royal Society of London Series B* **188,** 361–375.

Kachroo A, Nasrallah ME, Nasrallah JB. 2002. Self-incompatibility in the *Brassicaceae*: Receptor-ligand signaling and cell-to-cell communication. *The Plant Cell* **14** (suppl), S227–S238.

Kearns, CA, **Inouye, DW**, eds. 1993. *Techniques for Pollination Biologists*. University Press of Colorado, Niwot, CO.

Kermicle JL, Evans MMS. 2005. Pollen-pistil barriers to crossing in maize and teosinte result from incongruity rather than active rejection. *Sexual Plant Reproduction* **18,** 187–194.

Kessler SA, Shimosato-Asano H, Keinath NF, Wuest SE, Ingram G, Panstruga R, Grossniklaus U. 2010. Conserved molecular components for pollen tube reception and fungal invasion. *Science* **330,** 968–972.

Konar RN, Linskens HF. 1966. Physiology and biochemistry of the stigma fluid of *Petunia hybrida*. *Planta* **71,** 372–387.

Kondo K, Yamamoto M, Itahashi R, Sato T, Egashira H, Hattori T, Kowyama Y. 2002*a*. Insights into the evolution of self-compatibility in *Lycopersicon* from a study of stylar factors. *The Plant Journal* **30,** 143–153.

Kondo K, Yamamoto M, Matton DP, Sato T, Hirai M, Norioka S, Hattori T, Kowyama Y. 2002*b*. Cultivated tomato has defects in both *S-RNase* and *HT* genes required for stylar function of self-incompatibility. *The Plant Journal* **29,** 627–636.

Kuboyama T, Chung CS, Takeda G. 1994. The diversity of interspecific pollen-pistil incongruity in *Nicotiana*. *Sexual Plant Reproduction* **7,** 250–258.

Lewis D, Crowe LK. 1958. Unilateral interspecific incompatibility in flowering plants. *Heredity* **12,** 233–256.

Li W, Royer S, Chetelat RT. 2010. Fine mapping of ui6.1, a gametophytic factor controlling pollen-side Unilateral Incompatibility in interspecific *Solanum* hybrids. *Genetics* **185,** 1069–1080.

Liedl BE, McCormick S, Mutschler MA. 1996. Unilateral incongruity in crosses involving *Lycopersicon pennellii* and *L. esculentum* is distinct from self-incompatibility in expression, timing and location. *Sexual Plant Reproduction* **9,** 299–308.

Lin P, Xia L, Ng TB. 2007. First isolation of an antifungal lipid transfer peptide from seeds of a *Brassica* species. *Peptides* **28,** 1514–1519.

Linskens HF. 1975. Incompatibility in *Petunia*. *Proceedings of the Royal Society of London Series B* **188,** 299–311

Lowry DB, Modliszewski JL, Wright KM, Wu CA, Willis JH. 2008. The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical Transactions of the Royal Society of London Series B* **363,** 3009–3021.

Lush WM. 1999. Whither chemotropism and pollen tube guidance? *Trends in Plant Science* **4,** 413–418.

Marín-Rodríguez MC, Orchard J, Seymour GB. 2002. Pectate lyases, cell wall degradation and fruit softening. *Journal of Experimental Botany* **53,** 2115–2119.

Marshall E, Costa LM, Gutierrez-Marcos, J. 2011. Cysteine-Rich Peptides (CRPs) mediate diverse aspects of cell–cell communication in plant reproduction and development. *Journal of Experimental Botany* **62,** 1677–1686.

Matsuzaki T, Koiwai A, Kawashima N. 1983. Changes in stigma-specific lipids in tobacco plant during flower development. *Plant and Cell Physiology* **24,** 207–213.

McClure B. 2009. Darwin's foundation for investigating self-incompatibility and the progress toward a physiological model for S-RNase-based SI. *Journal of Experimental Botany* **60,** 1069–1081.

McClure BA, Franklin-Tong V. 2006. Gametophytic self-incompatibility: understanding the cellular mechanisms involved in "self" pollen tube inhibition. *Planta* **224,** 233–245.

McClure BA, Mou B, Canevascini S, Bernatzky R. 1999. A small asparagine-rich protein required for S-allele-specific pollen rejection in *Nicotiana*. *Proceedings of the National Academy of Sciences USA* **96,** 13548–13553.

McCubbin AG, Kao T-H. 2000. Molecular recognition and response in pollen-pistil interactions. *Annual Review of Cell and Developmental Biology* **16,** 333–364.

Murfett J, Bourque JE, McClure BA. 1995. Antisense suppression of S-RNase expression in *Nicotiana* using RNA polymerase II- and III-transcribed gene constructs. *Plant Molecular Biology* **29,** 201–212.

Murfett J, Strabaia JT, Zurek DM, Mou B, Beecher B, McClure BA. 1996. S RNase and interspecific pollen rejection in the genus *Nicotiana*: multiple pollen rejection pathways contribute to unilateral incompatibility between self-incompatible and self-compatible species. *The Plant Cell* **8,** 943–958.

Muschietti J, Dirchs L, Vancanneyt G, McCormick S. 1994. LAT52 protein is essential for tomato pollen development: pollen expressing antisense LAT52 RNA hydrates and germinates abnormally and cannot achieve fertilization. *The Plant Journal* **6,** 321–388.

Nakamura Y, Arimitsu H, Yamaryo Y, Awai K, Masuda T, Shimada H, Takamiya K, Ohta H. 2003. Digalactosyldiacylglycerol is a major glycolipid in floral organs of *Petunia hybrida*. *Lipids* **38,** 1107–1112.

Nakamura Y, Kobayashi K, Ohta H. 2009. Activation of galactolipid biosynthesis in development of pistils and pollen tubes. *Plant Physiology and Biochemistry* **47,** 535–539.

O'Brien M, Kapfer C, Major G, Laurin M, Bertrand C, Kondo K, Kowyama Y, Matton DP. 2002. Molecular analysis of the stylar-expressed *Solanum chacoense* asparagine-rich protein family related to the HT modifier of gametophytic self-incompatibility in *Nicotiana*. *The Plant Journal* **32,** 1–12.

Okuda S, Tsutsui H, Shiina K, Sprunck S, Takeuchi H, Yui R, Kasahara RD, Hamamura Y, Mizukami A, Susaki D, Kawano N, Sakakibara T, Namiki S, Itoh K, Otsuka K, Matsuzaki M, Nozaki H, Kuroiwa T, Nakano A, Kanaoka MM, Dresselhaus T, Sasaki N, Higashiyama T. 2009. Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. *Nature* **458,** 357–361.

Pandey KK. 1981. Evolution of unilateral incompatibility in flowering plants: further evidence in favour of twin specificities controlling intra- and interspecific incompatibility. *New Phytologist* **89,** 705–728.

Park SY, Lord EM. 2003. Expression studies of SCA in lily and confirmation of its role in pollen tube adhesion. *Plant Molecular Biology* **51,** 183–189.

Puerta AR, Ushijima K, Koba T, Sassa H. 2009. Identification and functional analysis of pistil-part self-incompatibility factor HT-B of *Petunia*. *Journal of Experimental Botany* **60,** 1309–1318.

R Development Core Team. 2010. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. www.R-project.org/.

Rick CM, Holle M, Thorp RW. 1978. Rates of cross-pollination in *Lycopersicon pimpinellifolium*: impact of genetic variation in floral characters. *Plant Systematics and Evolution* **129,** 31–44.

Rieseberg LH, Willis JH. 2007. Plant speciation. *Science* **317,** 910–914.

Sánchez AM, Bosch M, Bots M, Nieuwland J, Feron R, Mariani C. 2004. Pistil factors controlling pollination. *The Plant Cell* **16,** S98-S106.

Singh PK, Rathore SVS, Tripathi SK. 2004. Studies on self-incompatibility and heterotic response in sprouting broccoli (*Brassica oleracea* var. *italica* Plenck). *Acta Horticulturae* **706,** 179–187.

Sink KC, Power JB. 1978. Incongruity of interspecific and intergeneric crosses involving *Nicotiana* and *Petunia* species that exhibit potential for somatic hybridization. *Euphytica* **27,** 725–730.

Spooner DM, Peralta IE, Knapp S. 2005. Comparison of AFLPs with other markers for phylogenetic inference in wild tomatoes [*Solanum* L. section *Lycopersicon* (Mill.) Wettst.]. *Taxon* **54,** 43–61.

Stephenson AG, Good SV, Vogler DW. 2000. Interrelationships among inbreeding depression, plasticity in the self-incompatibility system, and the breeding system of *Campanula rapunculoides* L. (Campanulaceae). *Annals of Botany* **85,** 211–219.

Stotz HU, Spence B, Wang YJ. 2009. A defensin from tomato with dual function in defense and development. *Plant Molecular Biology* **71,** 131–143.

Tang W, Kelley D, Ezcurra I, Cotter R, McCormick S. 2004. LeSTIG1, an extracellular binding partner for the pollen receptor kinases LePRK1 and LePRK2, promotes pollen tube growth *in vitro*. *The Plant Journal* **39,** 343–353.

The Tomato Genome Consortium. 2012. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* **485,** 635–641.

Tung C, Dwyer KG, Nasrallah ME, Nasrallah JB. 2005. Genome-wide identification of genes expressed in Arabidopsis pistils specifically along the path of pollen tube growth. *Plant Physiology* **138,** 977–989.

Verhoeven T, Feron R, Wolters-Arts M, Edqvist J, Gerats T, Derksen J, Mariani C. 2005. STIG1 controls exudate secretion in the pistil of petunia and tobacco. *Plant Physiology* **138,** 153–160.

Waser NM, Ollerton J. 2006. *Plant–Pollinator Interactions: from Specialization to Generalization*. Chicago, University of Chicago Press.

Webb MC, Williams EG. 1988. Effects of temperature, light, nutrients and carbon dioxide on the strength of the self-incompatibility response in detached flowers of *Lycopersicon peruvianum*. *Annals of Botany (London)* **61,** 395–404.

Williams EG, Knox RB. 1982. Quantitative analysis of pollen tube growth in *Lycopersicon peruvianum*. *Journal of Palynology* **18,** 65–74.

Wise S, Reidegeld KA, Meyer HE, Warscheid B. 2007. Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research. *Proteomics* **7,** 340–350.

Wolters-Arts M, Mary Lush W, Mariani C. 1998. Lipids are required for directional pollen-tube growth. *Nature* **392,** 818–821.

Wu Y, Meeley RB, Cosgrove DJ. 2001. Analysis and expression of the α-expansin and β-expansin gene families in maize. *Plant Physiology* **126,** 222–232.

Yeats TH, Rose JKC. 2008. The biochemistry and biology of extracellular plant lipid-transfer proteins (LTPs). *Protein Science* **17,** 191–198.